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E-Selectin Is Present in Proliferating Endothelial Cells in Human Hemangiomas

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E-selectin, an endothelial-cell-specific leukocyte adhesion molecule, may also function in angiogenesis. To investigate its role in a noninflammatory angiogenic disease, E-selectin was analyzed by immunohistochemistry in specimens of proliferative phase and involutive phase hemangiomas. Hemangioma is an endothelial cell tumor of capillary blood vessels that grows rapidly during infancy and regresses spontaneously during childhood. E-selectin expression was high in proliferative phase specimens and was co-localized with dividing microvascular endothelial cells. Relative to the number of blood vessels, E-selectin declined significantly in involutive phase specimens demonstrating that E-selectin correlates with angiogenesis in the tumors. E-selectin was not detected in quiescent endothelium but was co-localized in dividing microvascular endothelial cells in placenta and neonatal foreskin, two tissues with ongoing growth of microvessels. These* in vivo *studies support the hypothesis that E-selectin functions in angiogenesis and suggest that E-selectin may be a marker for proliferating endothelium. (Am J Pathol 1996, 148:1181–1191)

Angiogenesis, the formation of new capillaries from pre-existing microvessels, is a tightly regulated process during fetal growth and development.[1] In adults, the endothelium is quiescent, and turnover of endothelial cells is extremely slow.[2] Exceptions are the growth of microvessels during the female reproductive cycle or during wound healing and inflammation. Angiogenesis also occurs in many diseases such as atherosclerosis, rheumatoid arthritis, diabetic retinopathy, and tumor growth.[3] Angiogenesis is a multi-step process in which endothelial cells degrade their extracellular matrix, migrate into the perivascular spaces, proliferate, and align to form cell-cell contacts to construct patent blood vessels. The discovery of endogenous stimulators of angiogenesis, such as basic fibroblast growth factor and vascular endothelial growth factor, and inhibitors of angiogenesis, such as tissue inhibitors of metalloproteases, angiostatin, and thrombospondin, has led to the hypothesis that angiogenesis is controlled by a balance of angiogenic stimulators and inhibitors.[3] However, there are many unanswered questions about angiogenesis and its role in human disease.

Less is known about the molecules that mediate the cell-cell interactions required for the alignment of endothelial cells into capillary tubes. Two membrane-bound cell adhesion molecules have been implicated. Brooks et al[4] found that the integrin $\alpha_v\beta_3$ is required for angiogenesis induced by basic fibroblast growth factor or tumor in the chick chorioallantoic membrane assay. Using an *in vitro* assay in which endothelial cells are induced to form capillary-like tubes, Nguyen et al[5] found that E-selectin, an endothelial-cell-specific membrane glycoprotein, participates in capillary tube formation. More recently, a soluble form of E-selectin was found to stimulate neovascularization in the rat cornea.[6] These studies suggest that, in addition to its function in leukocyte adhesion, E-selectin is involved in the formation of capillary blood vessels.

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E-selectin was first identified as a cytokine-inducible antigen in human umbilical cord vein endothelial cells[7] and subsequently shown to participate in the rolling of myeloid cells along endothelium at sites of inflammation.[8] *In vivo*, E-selectin expression is thought to be restricted to post-capillary venules at sites of inflammation or immune activation and absent in quiescent endothelium.[9] The selectins, a family of leukocyte adhesion molecules that includes P- and L-selectin, are calcium-dependent carbohydrate-binding proteins that recognize sialylated, fucosylated oligosaccharides and related structures.[10] To further elucidate selectin function in leukocyte trafficking, mice deficient in either E-, P-, or L-selectin have been generated by targeted gene disruption.[11–13] A defect in neutrophil extravasation was detected in E-selectin-deficient mice after P-selectin function was blocked indicating that E- and P-selectin serve overlapping functions in endothelium.[11] The viability of E-selectin-deficient mice suggests that E-selectin is not required for angiogenesis during murine development. This is perhaps not surprising, for given the crucial role the microvasculature plays in organ development and function, it is likely that redundant mechanisms have evolved to ensure that angiogenesis occurs at precise times and locations.

To learn more about the function of E-selectin in growing microvessels *in vivo*, we analyzed E-selectin by immunohistochemistry in three noninflammatory human tissues: hemangioma, placenta, and neonatal foreskin. Hemangiomas, the most common tumors of infancy, represent examples of pure angiogenesis composed of growing capillary blood vessels. Hemangiomas typically appear soon after birth and grow rapidly during the first year of life. The tumors begin to regress between 12 and 24 months, although proliferation continues simultaneously with involution, as assessed by autoradiography of [^{3}H]thymidine-labeled specimens and clinical observations.[14] Involution predominates after 2 years of age. Some hemangiomas grow dramatically, causing either deformation, destruction, or obstruction of the surrounding tissue, and large hemangiomas can cause life-threatening complications before the involutive phase begins, ie, thrombocytopenia (Kasabach-Merritt phenomenon) or high-output congestive heart failure.

We found that tissues with actively growing microvessels, hemangioma, placenta, and neonatal foreskin, express E-selectin polypeptide that is colocalized with dividing endothelial cells. In involutive phase hemangiomas, E-selectin declined significantly relative to number of blood vessels, indicating that E-selectin expression correlates with the angiogenic phenotype of the hemangioma. These findings demonstrate that E-selectin is expressed in proliferating endothelial cells and provide the first *in vivo* localization of E-selectin in angiogenic blood vessels. These data support the hypothesis that E-selectin functions in angiogenesis. Furthermore, E-selectin may be a useful *in vivo* marker for proliferating (angiogenic) endothelial cells.

Materials and Methods

Materials

Materials were obtained from the following sources: Superfrost/Plus slides from Fisher Scientific (Springfield, NJ); *Ulex europaeus* -I, anti-Ki-67 MM1 (catalog number NCL-Ki-67-MM1), biotinylated horse anti-mouse IgG (catalog number BA2000), biotinylated goat anti-rabbit IgG (catalog number BA1000), horseradish peroxidase (HRP)-conjugated horse anti-mouse IgG (catalog number PI 2000), and the diaminobenizidine (DAB) substrate kit from Vector Laboratories (Burlingame, CA); avidin/biotin-HRP detection kit from Pierce (Rockford, IL); purified mouse IgG1 (catalog number 550029) and anti-human P-selectin (catalog number 550014) from Becton Dickinson (San Jose, CA); Gamma-bind Plus from Pharmacia (Piscataway, NJ); goat anti-mouse IgG-Sepharose 4B from Cappel Laboratories (Durham, NC); tumor necrosis factor (TNF)-α from Boehringer Mannheim, (Indianapolis, IN); tosyl-activated magnetic Dynabeads (M-450) from Dynal (Great Neck, NY); gelatin from Difco Laboratories (Detroit, MI); and [^{35}S]cysteine (specific activity, 1000 Ci/mmole) from NEN-DuPont (Wilmington, DE). The cDNAs for human E-, P-, and L-selectin, subcloned in the plasmid pMT2, were provided by Dr. G. Kansas (Northwestern University School of Medicine, Chicago, IL). Anti-CD31 polyclonal rabbit serum[15] was provided by Dr. Steven Albelda (University of Pennsylvania, Philadelphia, PA). Anti E-selectin mouse monoclonal antibody (MAb) HAE-1e was provided by Dr. T. Tedder (Duke University, Durham, NC). Human E-selectin-Ig was provided by Dr. D. Hollenbaugh (Bristol-Myers Squibb Pharmaceutical Research Institute).

Collection of Tissue Specimens for This Study

Tissue specimens of hemangioma and vascular malformation were obtained immediately after surgical excision. Third-trimester placental samples were ob-

tained after delivery by cesarean section. Neonatal foreskins were collected immediately after circumcision. Normal adult human skin specimens were 5-mm punch biopsies of volunteer donors. Tissue collection was performed according to procedures approved by the Internal Review Board. Specimens were embedded in OCT compound, frozen on dry ice, and stored at −80°C.

Antibodies Used for Immunohistochemistry

A human E-selectin-Ig fusion protein, constructed as described for P-selectin,[16] was used to generate anti-human E-selectin mouse MAbs. One hybridoma, designated 5G11, produced an IgG1 that reacted with E-selectin-Ig and cytokine-activated human umbilical vein endothelial cells. 5G11 was purified using Gamma-bind Plus and assayed for cross-reactivity with human P-selectin. No binding to P-selectin was observed using an enzyme-linked immunosorbent assay with a P-selectin-Ig or by FACS analysis of activated platelets. Sources of other antibodies are listed under Materials.

Immunohistological Examination of Tissues

Five-micron frozen sections of tissues were placed onto Superfrost/Plus slides, fixed in ice-cold acetone for 1 to 2 minutes, and stored at −80°C. Before immunostaining, sections were re-fixed in ice-cold acetone for 15 minutes. For staining of nuclear antigens, sections were permeabilized in 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 20 minutes. Endogenous peroxidase was inactivated with 0.3% H_2O_2 in PBS for 30 minutes. Nonspecific binding sites were blocked with 5% horse serum in PBS. The 5G11 MAb (5 μg/ml), Ki-67 MAb (1:200 dilution), isotype-matched control MAb (5 μg/ml), rabbit anti-CD31 (1:5000 dilution), and preimmune rabbit serum (1:5000 dilution) were added for 2 hours at room temperature in a humidified chamber. Secondary biotinylated anti-mouse or anti-rabbit IgGs (7.5 μg/ml) were added for 1 hour. According to the manufacturer's instructions, a 1:100 dilution of the avidin/biotin-conjugated HRP reagent was incubated with the tissue sections for 30 minutes. The DAB substrate was then added for 5 to 7 minutes until a reddish-brown precipitate was visible. Tissues were counterstained with Gill's hematoxylin and treated with 30 mmol/L NH_4OH to generate a blue nuclear stain. Slides were dehydrated through an alcohol and xylene gradient and mounted with Permount glue. To analyze co-expression of Ki-67 and E-selectin, tissue sections were first incubated with a 1:100 dilution of anti-Ki-67 antibody, followed by HRP-conjugated anti-mouse IgG (5 μg/ml). Nickel (2 drops) was included in the DAB substrate to form a black precipitate. Specimens were reblocked with 5% horse serum for 30 minutes and incubated with the anti-E-selectin antibody (5 μg/ml), followed by biotinylated anti-mouse IgG and then avidin/biotin-conjugated HRP. The DAB reaction was performed in the absence of nickel to generate a reddish-brown cytoplasmic membrane precipitate that could be distinguished easily from Ki-67-stained nuclei.

Quantitation and Analysis of the Immunolabeled Frozen Sections

Three representative fields were selected in each tissue section at ×400 magnification. CD31- and E-selectin-positive microvessels and Ki-67-positive nuclei were counted in each field; numbers were averaged and expressed as CD31- and E-selectin-positive vessels/0.16 mm^2 ± SD and as Ki-67-positive cells/0.16 mm^2 ± SD. The number of E-selectin-positive vessels/0.16 mm^2 for each specimen was expressed as a percentage of CD31-positive vessels based on the following formula: (absolute counts for E-selectin/absolute counts for CD31) × 100 = percentage of E-selectin-positive vessels.

To assess the dividing cells per microvessel in hemangioma specimens, the Ki-67-positive endothelial cells were also normalized to the total CD31-positive endothelium. CD31, E-selectin, and Ki-67 counts were performed independently by three observers. One set of these results is presented for each tissue. There was excellent agreement among the three observers.

Statistical Analysis

Analysis of the data consisted of two methods. First, decreasing E-selectin-positive microvessels and Ki-67-positive endothelial cells with age, both expressed as a percentage of total CD31-positive microvessels, were analyzed using linear regression models. Second, the hemangioma specimens were divided into two groups according to age: group A included specimens from patients younger than 24 months and group B included specimens from patients 24 months and older. The E-selectin/CD31 and Ki-67/CD31 ratios were compared between these two age groups using Student's *t*-test. Data are expressed in terms of mean values (±SD). All *P* values reported are two-tailed with a type I error rate of 0.05 used for all analysis. Statistical calculations were

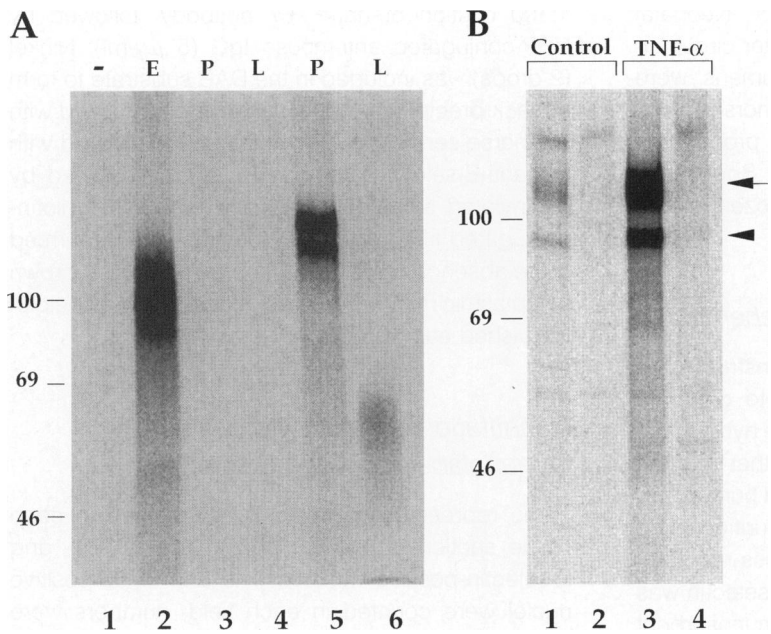

Figure 1. *Specificity of anti-human E-selectin MAb 5G11.* A: *COS cells were transfected with no DNA* (lane 1) *or cDNAs for human E-selectin* (lane 2), *P-selectin* (lanes 3 *and* 5), *and L-selectin* (lanes 4 *and* 6), *metabolically labeled with* [^{35}S]*cysteine and immunoadsorbed with anti-E-selectin MAb 5G11* (lanes 1 *to* 4), *anti-P-selectin* (lane 5), *or anti-L-selectin* (lane 6). B: *Expression of E-selectin in HDMECs. HDMECs either unstimulated* (lanes 1 *and* 2) *or stimulated with TNF-α for 4 hours* (lanes 3 *and* 4) *were labeled with* [^{35}S]*cysteine for 2 hours and immunoadsorbed with MAb 5G11* (lanes 1 *and* 3) *or control mouse IgG1* (lanes 2 *and* 4). *In* A *and* B, *immune complexes were subjected to SDS-polyacrylamide gel electrophoresis and phosphorimager analysis. Molecular weight markers are indicated in the left margin. In* B, *the* arrow *denotes the 115-kd mature E-selectin and the* arrowhead *denotes the 97-kd E-selectin precursor.*

performed with the SAS Statistical Package (Version 6.10, SAS Institute, Cary, NC).

Specificity of the E-Selectin MAb 5G11

COS-7 cells were transfected[17] with plasmids containing cDNAs encoding human E-, P-, and L-selectin, labeled for 2 hours with [^{35}S]cysteine, lysed, precleared,[18] and incubated with 10 μg/ml 5G11 MAb overnight on ice, adsorbed with 50 μl of a 1:1 suspension of goat anti-mouse IgG-Sepharose for 2 hours at 4°C, washed, and subjected to 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.[18] To verify the expression of P- and L-selectin, an equivalent portion of cell lysate from P-selectin and L-selectin transfectants were immunoadsorbed with anti-P-selectin or anti-L-selectin. [^{35}S]Cysteine-labeled proteins were visualized by phosphorimager analysis.

Endothelial Cell Culture

Human dermal microvascular endothelial cells (HDMECs) were isolated from neonatal foreskin and purified from contaminating cells using *Ulex europaeus*-I-coated magnetic Dynabeads M-450.[19,20]

Immunoadsorption of E-Selectin from HDMECs

HDMECs (passage 7) in 60-mm dishes were cysteine starved for 2 hours in medium devoid of cysteine. TNF-α (200 U/ml) was added to one dish at the time of cysteine starvation. Cells were labeled with 150 μCi/ml [^{35}S]cysteine for 2 hours, lysed in 500 μl of 25 mmol/L Tris-HCl, pH 7.4, 2% NP-40, 0.1 mmol/L leupeptin, 12.5 μg/ml aprotinin, and precleared with 250 μl of a 1:1 suspension of Sepharose CI-4B. Anti-E-selectin MAb 5G11 and control IgG1 antibodies were used at 10 μg/ml. Immunoadsorption and analysis were performed as described above.

Results

Characterization of Anti-E-Selectin Antibodies

An anti-human E-selectin MAb, designated 5G11, was assayed for its reactivity with frozen sections of human hemangiomas by immunohistochemistry and for potential cross-reactivity with P-selectin, a homologous protein constitutively expressed in endothelium, and L-selectin, the third member of the selectin family expressed in leukocytes.[8] First, COS-7 cells were transfected with cDNAs for either human E-, human P-, or human L- selectin, metabolically labeled with [^{35}S]cysteine, and immunoadsorbed with 5G11 MAb (Figure 1A). The anti-E-selectin MAb reacted specifically with E-selectin (lane 2) and did not cross-react with human P- or L-selectin (lanes 3 and 4) or mock-transfected cells (lane 1) indicating that 5G11 is specific for human E-selectin. COS cells transfected with P- or L-selectin cDNAs were shown

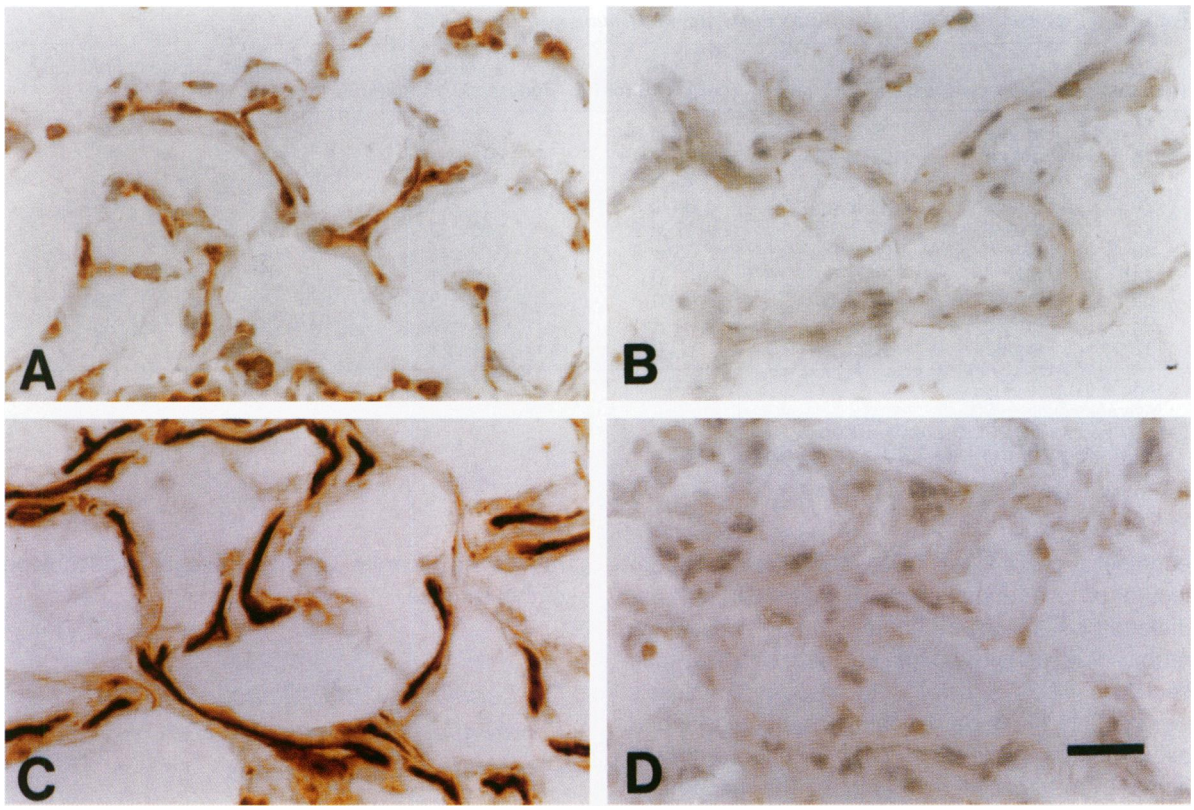

Figure 2. *E-selectin expression in proliferative phase hemangioma. Indirect immunohistochemistry was performed on 5-μm frozen sections with biotinylated secondary antibodies, avidin/biotin-conjugated HRP, and DAB substrate.* **A:** *Staining with anti-E-selectin.* **B:** *Anti-E-selectin was preadsorbed with a 10-fold excess of human E-selectin-Ig (50 μg/ml) for 1 hour before immunostaining.* **C:** *Staining with anti-CD31.* **D:** *Background staining observed with control mouse IgG1. Magnification, ×630; bar, 25 μm.*

to be producing P-selectin (lane 5) and L-selectin (lane 6) polypeptides as demonstrated by immunoadsorption using antibodies directed against human P- and L-selectin.

In a second assay, the 5G11 MAb was tested for immunoadsorption of E-selectin synthesized in HDMECs. Unstimulated and TNF-α-stimulated subconfluent HDMEC cultures were immunoadsorbed with anti-human E-selectin MAb (Figure 1B). E-selectin was expressed at low levels in unstimulated growing HDMEC cultures (lane 1), consistent with previous reports of mRNA expression in bovine capillary endothelial cells[5,21] and human umbilical vein endothelial cells.[22] As expected, the level of E-selectin was increased by cytokine stimulation (lane 3).[7] The arrow denotes the 115-kd mature E-selectin and the arrowhead denotes the 97-kd E-selectin precursor detected under these metabolic labeling conditions. 5G11 MAb did not react with the 140-kd P-selectin, which further demonstrated the specificity of 5G11 for human E-selectin. No reactivity was observed with nonspecific mouse IgG1 control MAb (lanes 2 and 4). Because of these findings, the 5G11 MAb was used for immunohistological analysis of E-selectin expression.

Immunohistochemical Analysis of Human E-Selectin in Hemangiomas

Frozen sections of 10 different hemangioma specimens (Table 1) were examined for expression of endothelial cell antigens CD31, E-selectin, and P-selectin. CD31, also known as platelet endothelial cell adhesion molecule-1, is ubiquitously expressed by continuous endothelium[15] and is a reliable endothelial cell marker for quantitation of microvessel density in breast carcinoma biopsies.[23]

E-selectin was detected along the microvascular endothelium in hemangioma specimens. Staining of a 20-month-old proliferative phase hemangioma (Table 1) is shown in Figure 2. Immunoreactive E-selectin was detected in many microvessels (Figure 2A). Binding of 5G11 MAb to the tissue section was abolished by preincubation of 5G11 MAb with 10-fold excess of soluble E-selectin (Figure 2B). The

Table 1. *Expression of CD31, Ki67, and E-selectin in Hemangioma Specimens*

Age* (months)	Gender	CD31† (vessels/0.16 mm²)	Ki67† (cells/0.16 mm²)	E-selectin† (vessels/0.16 mm²)	Ki67/CD31‡ (%)	E-selectin/CD31§ (%)
5	Female	23 ± 3	12 ± 2	18 ± 2	52	78
8	Female	107 ± 23	69 ± 18	93 ± 12	64	87
18	Female	74 ± 23	71 ± 15	64 ± 11	96	86
20	Female	31 ± 6	34 ± 5	33 ± 10	110	106
24	Male	44 ± 12	17 ± 2	15 ± 2	39	34
40	Male	26 ± 6	5 ± 3	12 ± 1	19	46
44	Male	30 ± 6	0	3 ± 1	0	10
66	Female	33 ± 9	0	18 ± 3	0	55
93	Female	16 ± 4	1 ± 1	4 ± 4	6	25
112	Female	10 ± 3	0	1 ± 1	0	10

*Age at the time of excision.
†Average counts ± SD.
‡Ki67-positive cells/CD31-positive vessels × 100.
§E-selectin-positive vessels/CD31-positive vessels × 100.

endothelial cell antigen CD31 was also readily detected on the endothelium (Figure 2C). Isotype-matched control IgG1 (Figure 2D) and preimmune rabbit serum (not shown) did not react with the tissue sections. CD31- and E-selectin-positive vessels were counted in three optical fields, averaged, and expressed as vessels/0.16 mm² for each of the 10 hemangioma specimens (Table 1). The mean number of CD31-positive vessels was slightly higher in proliferative phase hemangiomas (n = 4) compared with hemangioma specimens from children older than 24 months (n = 6; $P = 0.09$), consistent with previous studies.[24]

E-selectin immunoreactivity was most prominent in proliferative phase hemangiomas wherein 78 to 100% of the microvessels stained positively for E-selectin (Table 1). E-selectin-positive vessels, expressed as a percentage of CD31-positive vessels, decreased with increasing age of the children, corresponding with progression of involution (linear regression on age, $P = 0.014$). Student's *t*-test analysis showed that proliferative phase hemangioma specimens (patients younger than 24 months) expressed significantly higher levels of E-selectin relative to CD31 than involutive phase hemangiomas (patients older than 24 months; $P < 0.001$). Thus, the proliferative phase, during which rapid vessel growth and angiogenesis occur, is characterized by E-selectin expression in most vessels whereas diminishing E-selectin coincides with regression of the tumor. These findings were confirmed with a second anti-human E-selectin MAb, HAE-1e (not shown). The percentage of P-selectin-positive vessels varied from 50 to 90% among hemangioma specimens, but a difference between proliferating and involuting tissues was not observed (not shown). Thus, unlike CD31 or P-selectin, the presence of E-selectin correlates significantly with the angiogenic phenotype of this disease.

Co-Localization of E-Selectin and the Proliferating Cell Marker Ki-67 in Proliferating Hemangiomas

Hemangioma specimens were examined for proliferating endothelial cells by staining with an anti-Ki-67 MAb (Figure 3). The Ki-67 antigen is expressed during G1, S, and G2 phases of the cell cycle but absent in resting (G0) cells.[25] The Ki-67-positive nuclei were counted and expressed as Ki-67-positive nuclei/0.16 mm². Proliferative phase hemangiomas were characterized by hyperplasia (Figure 3A), which declined during involution (Table 1).

Double labeling of the hemangioma frozen sections for Ki-67 and E-selectin was performed by incubating frozen sections first with anti-Ki-67 MAb and then with anti-E-selectin MAb (Figure 3B). The Ki-67 labeling technique produced a black precipitate in nuclei whereas anti-E-selectin labeling produced a reddish-brown cytoplasmic membrane precipitate. Most of the dividing cells (black nuclei) co-localized with the E-selectin-positive endothelium (reddish brown) (Figure 3B). For comparison, immunostaining for E-selectin alone is shown in Figure 3C. Localization of E-selectin in dividing endothelial cells indicates that E-selectin expression *in vivo* is coincident with endothelial cell proliferation. Thus, E-selectin may be considered an endothelial proliferation antigen as well as an activation antigen.

The number of Ki-67-positive cells was expressed as a percent of CD31-positive endothelium to obtain a ratio of dividing cells per microvessel (Table 1). Hemangiomas from patients younger than 24

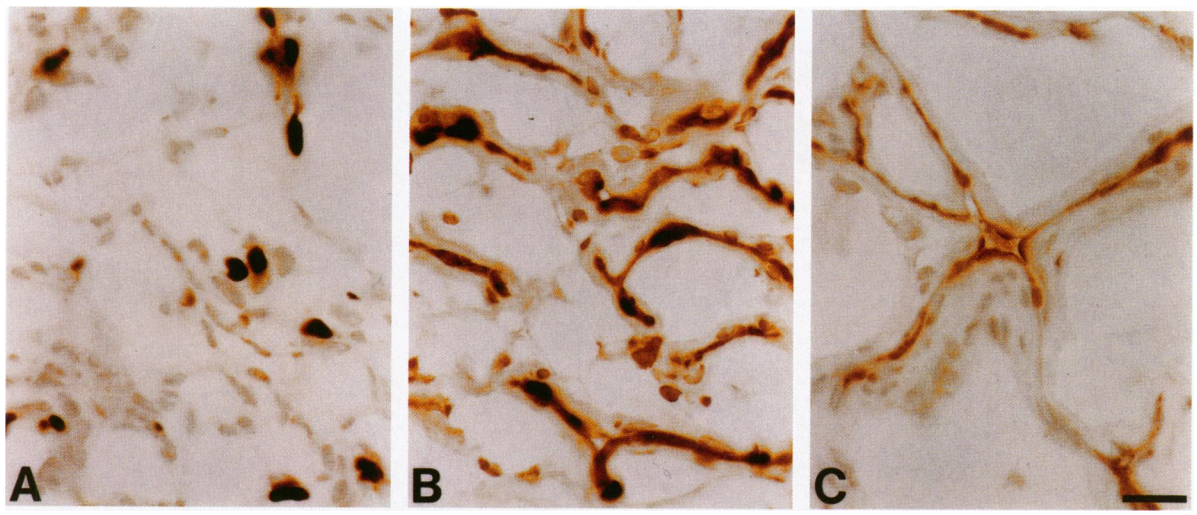

Figure 3. *Double labeling of a proliferating hemangioma with anti-E-selectin and anti-Ki-67 MAbs.* **A:** *Five-micron frozen sections immunolabeled with anti-Ki-67.* **B:** *For double labeling, anti-Ki-67 was performed first using nickel in the DAB substrate to produce a black reaction product. Immunostaining with anti-E-selectin was carried out with DAB alone to produce a reddish-brown reaction product.* **C:** *Immunostaining with anti-E-selectin alone. Magnification, ×630; bar, 25 μm.*

months contained significantly more dividing endothelial cells per microvessel than specimens from older children ($P < 0.001$). The numbers of dividing endothelial cells diminished significantly with age (linear regression on age; $P = 0.019$), and Ki-67-positive nuclei were not detected in most of the involuted specimens. The decrease in numbers of dividing endothelial cells coincided with the decrease in E-selectin-positive microvessels.

E-Selectin in Human Angiogenic and Non-Angiogenic Tissues

We also analyzed third-trimester placenta and normal neonatal foreskin for E-selectin, CD31, and Ki-67 (Figure 4). Biopsies of vascular malformations and normal adult human skin were examined as examples of non-angiogenic tissue (Figure 4 and Table 2). Quantitation of microvessel density, E-selectin expression, and proliferating cells was performed as described above.

Placenta is a physiological tissue characterized by high microvessel density and angiogenesis that occurs throughout gestation.[26] The placental microvessels stained positive for CD31 (Figure 4A), E-selectin (Figure 4B), and Ki-67 (Figure 4C). Co-localization of Ki-67-positive nuclei and E-selectin-positive endothelial cells was also observed (not shown). However, the six placental specimens analyzed displayed great variability in the levels of E-selectin and Ki-67. The quantitative analysis was carried out on four of the placental specimens (Table 2).

Neonatal foreskin was chosen because its rapid growth suggests angiogenesis and it is a readily available source of human microvascular endothelial cells for *in vitro* studies. Neonatal foreskin (n = 6) contained higher CD31-positive microvessel densities (Figure 4D) than adult human skin (n = 3; Table 2). The microvascular endothelial cells in neonatal foreskin expressed E-selectin (Figure 4E; Table 2), and Ki-67-positive cells were found in the dermal microvessels (Figure 4F; Table 2). Dividing cells within the basal epidermis and dermal appendages (hair follicles and sebaceous and sweat glands) were not quantitated.

Vascular malformations are a heterogeneous group of structural vascular anomalies.[14,27] The

Table 2. *Expression of CD31, Ki67, and E-selectin in Normal Tissues*

Tissue	CD31* (vessels/0.16 mm^2)	Ki67* (cells/0.16 mm^2)	E-selectin* (vessels/0.16 mm^2)	E-selectin/CD31† (%)
Placenta	30 ± 17	12 ± 13	5 ± 4	17
Neonatal foreskin	14 ± 6	3 ± 2	3 ± 3	21
Adult human skin	5 ± 2	0	0	0

*Average counts ± SD.
†E-selectin-positive vessels/CD31-positive vessels × 100.

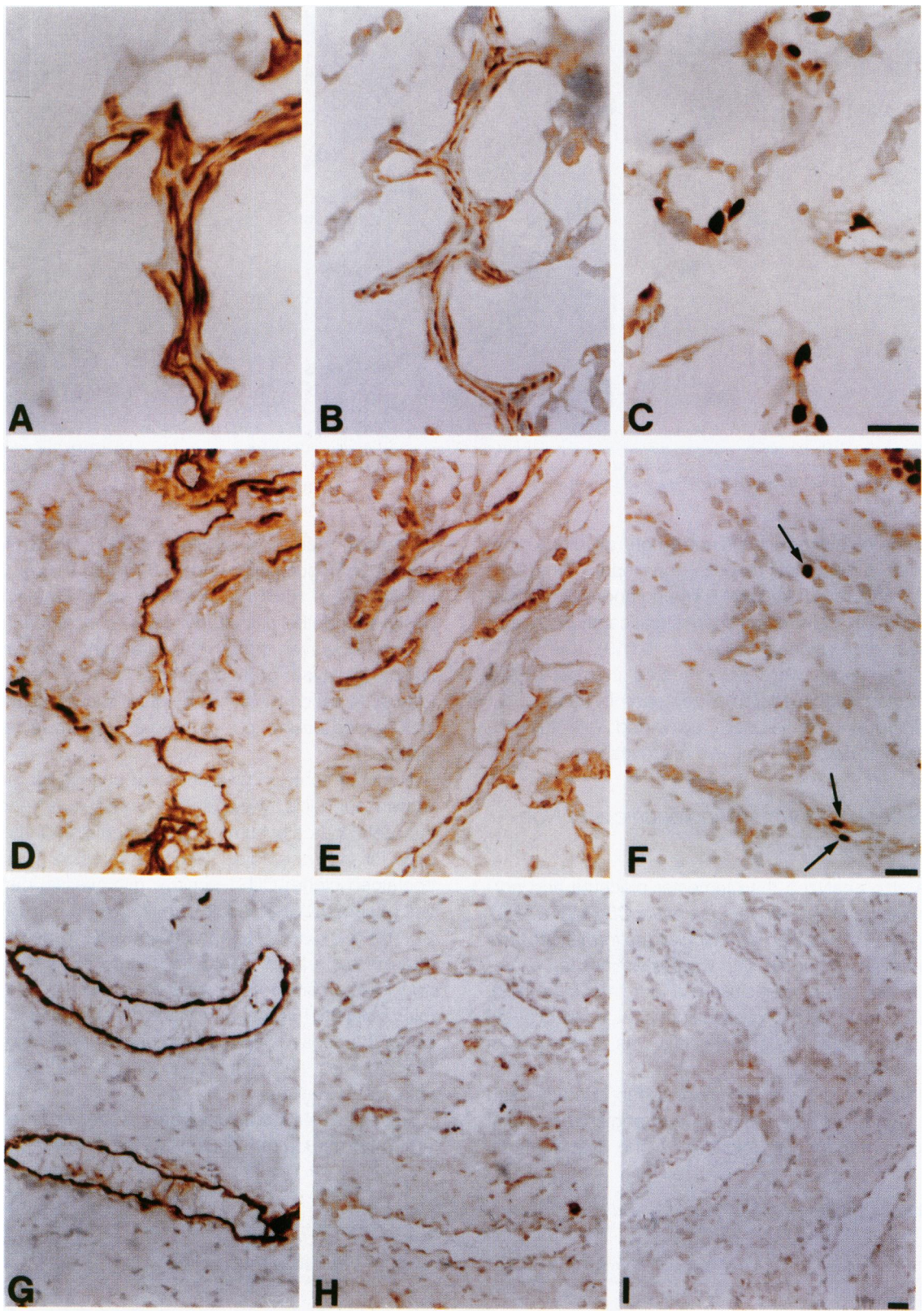

Figure 4. *CD31, E-selectin, and Ki-67 expression in human tissues. Five-micron frozen sections of third-trimester placenta* (A *to* C), *neonatal foreskin* (D *to* F), *and a venous malformation* (G *to* I) *immunolabeled with anti-CD31* (A, D, *and* G), *anti-E-selectin* (B, E, *and* H), *and anti-Ki-67* (C, F, *and* I). *The* arrows *in* F *mark proliferating cells located in dermal microvessels. Magnifications,* ×*630* (A *to* C), ×*400* (D *to* F), *and* ×*250* (G *to* I); *bars, 25* μ*m.*

most common are venous malformations characterized by enlarged and/or dilated thin-walled channels that lack an integrated layer of smooth muscle cells. In contrast to hemangiomas, vascular malformations grow proportionally with a child, they do not spontaneously regress, and endothelial proliferation is not detected.[14] Two venous malformations (slow flow), two arteriovenous malformations (fast flow), and one capillary malformation were qualitatively assessed for the expression of CD31, E-selectin, and Ki-67. Photomicrographs of a small venous malformation are shown in Figure 4, G–I. The endothelial cell lining of the malformed vascular channel expressed the CD31 antigen (Figure 4G). However, E-selectin expression was very low (Figure 4H) and Ki-67-positive proliferating cells were absent (Figure 4I). For adult human skin (n = 3), microvessels were detected with antibodies to CD31, none of which contained E-selectin or Ki-67 (Table 2). The absence of E-selectin expression in adult human skin is consistent with previous studies.[9]

Discussion

A previous study suggested that E-selectin functions during angiogenesis because formation of capillary tubes *in vitro* was inhibited by antibodies directed against E-selectin or its carbohydrate ligands sialyl Lewis-X and sialyl Lewis-A.[5] Recently, a soluble form of E-selectin added exogenously has been shown to induce neovascularization in the cornea.[6] To further investigate E-selectin function in the microvasculature *in vivo*, we analyzed human angiogenic and non-angiogenic tissues for the presence of E-selectin. E-selectin expression is known to be induced by inflammatory stimuli,[7,8] and therefore, angiogenic tissues without signs of inflammation, such as leukocytic infiltration and edema, were selected for this study. The tissues analyzed were hemangioma, neonatal foreskin, and third-trimester placenta. For comparison, two non-angiogenic tissues, adult human skin and vascular malformations, were also examined. We found that hemangioma, placenta, and neonatal foreskin specimens contained endothelial cells positive for both E-selectin and Ki-67 whereas the expression of both antigens was extremely low or undectable in non-angiogenic tissues. These studies demonstrate that E-selectin is expressed in dividing endothelial cells *in vivo* and thereby support the hypothesis that E-selectin functions during angiogenesis.[5,6] E-selectin-deficient mice, however, do not show obvious defects of vasculogenesis or angiogenesis as these mice breed and develop normally.[11] As angiogenesis is an important step in development, capillary blood vessel growth may be regulated by redundant mechanisms that can compensate for the lack of E-selectin in these mice.

Takahashi et al[24] analyzed hemangioma specimens for endothelial cell markers CD31 and von Willebrand factor and for a proliferating cell nuclear antigen, basic fibroblast growth factor, vascular endothelial cell growth factor, collagenase IV, and tissue inhibitor of metalloproteases. Proliferative phase hemangiomas exhibit high density of microvessels and proliferating cell nuclear antigen-positive cells whereas involuted hemangiomas contain relatively few blood vessels and proliferating cells. In their study, immunoreactivity was quantitated by counting one microscopic field that contained the highest density of immunolabeled structures and expressed as absolute value per surface area. In the present study, three representative areas from each section were selected and quantitated. We normalized the counts for E-selectin and Ki-67 to the CD31-positive vessel density, which facilitated analysis of E-selectin and Ki-67 relative to microvessel density within the specimen.

We found that hemangiomas in children younger than 24 months (proliferative phase) displayed increased E-selectin relative to microvessel density, compared with the older specimens (involutive phase; $P < 0.001$). Furthermore, significantly more dividing cells per CD31-positive vessel were found in these proliferative phase hemangiomas compared with involuting specimens older than 23 months ($P < 0.001$). The decline in E-selectin and Ki-67 correlates with diminishing angiogenesis and the onset of involution, and this may provide clues to the control of angiogenesis.

The mechanisms that regulate expression of E-selectin in hemangiogenesis and regression are unknown. There is no histological evidence for acute or chronic inflammation during the life cycle of hemangioma. However, late proliferative phase and involutive phase hemangiomas contain mast cells,[27] which are known to contain TNF-α.[28] Furthermore, mast-cell-derived TNF-α can induce E-selectin in dermal explants.[28] Thus, resident mast cells may release TNF-α, which may induce E-selectin expression in the microvascular endothelium of proliferating hemangiomas. Besides its proinflammatory activity, TNF-α has been shown to be angiogenic *in vivo*.[29–32] However, the role of TNF-α in hemangiomatous progression is not yet known.

E-selectin could be a valuable pathological marker for the detection of angiogenic microvessels. Recent studies using CD31 or von Willebrand factor

as endothelial markers have shown that intratumor microvessel density is an independent prognostic indicator for survival of invasive breast carcinoma.[23,33] Microvessel density has also been correlated with metastasis of other tumors.[34] Measurement of E-selectin, as a marker for angiogenic microvessel density, could increase sensitivity in these prognostic studies, because, as shown here, it is expressed in proliferating microvessels whereas CD31 is expressed in all vessels. E-selectin has been detected in tumor microvessels in cutaneous squamous cell carcinoma, basal cell carcinoma,[35] and colorectal carcinoma.[36] Weak expression of E-selectin in the human decidua during early pregnancy has been reported.[37] This observation is pertinent to our study as angiogenesis occurs at the endometrial implantation site. Immunohistochemical analysis of healthy, noninflammed gingival tissue also revealed E-selectin in microvessels in the connective tissue subjacent to junctional and oral epithelia,[38] although it is not known whether normal gingival tissue is a site of angiogenesis.

In summary, E-selectin is present in microvessels that contain proliferating endothelial cells, a hallmark of angiogenesis. Whether the expression of E-selectin in proliferative phase hemangioma, placenta, and foreskin is required for angiogenesis in human tissues is unknown. However, these observations support previously reported *in vitro* data suggesting a role for E-selectin and its sialylated, fucosylated ligands during capillary blood vessel growth.[5] Future studies will be directed toward analysis of E-selectin function in cytokine-induced angiogenesis and regulation of E-selectin by angiogenic factors.

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